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YybT Is a Signaling Protein That Contains a Cyclic Dinucleotide Phosphodiesterase Domain and a GGDEF Domain with ATPase Activity*[S]

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The cyclic dinucleotide c-di-GMP synthesized by the diadenylate cyclase domain was recently discovered as a messenger molecule for signaling DNA breaks in *Bacillus subtilis*. By searching bacterial genomes, we identified a family of DHH/DHHA1 domain proteins (COG3387) that co-occur with a subset of the diadenylate cyclase domain proteins. Here we report that the *B. subtilis* protein YybT, a member of the COG3387 family proteins, exhibits phosphodiesterase activity toward cyclic dinucleotides. The DHH/DHHA1 domain hydrolyzes c-di-AMP and c-di-GMP to generate the linear dinucleotides 5′-pApA and 5′-pGpG. The data suggest that c-di-AMP could be the physiological substrate for YybT given the physiologically relevant Michaelis-Menten constant (K_m) and the presence of YybT family proteins in the bacteria lacking c-di-GMP signaling network. The bacterial regulator ppGpp was found to be a strong competitive inhibitor of the DHH/DHHA1 domain, suggesting that YybT is under tight control during stringent response. In addition, the atypical GGDEF domain of YybT exhibits unexpected ATPase activity, distinct from the common diguanylate cyclase activity for GGDEF domains. We further demonstrate the participation of YybT in DNA damage and acid resistance by characterizing the phenotypes of the Δ*yybT* mutant. The novel enzymatic activity and stress resistance together point toward a role for YybT in stress signaling and response.

The cyclic dinucleotide c-di-GMP[2] has been firmly established as a major bacterial messenger molecule in recent years, with the cellular level of c-di-GMP regulated by diguanylate cyclase and phosphodiesterase domain proteins (1–3). In contrast, the existence of the structurally similar 3′,5′-cyclic diguanylate (c-di-AMP) in living cells was unknown until the recent serendipitous discovery of the dinucleotide bound by the DisA protein from *Bacillus subtilis* (4, 5). It was found that c-di-AMP was synthesized by the diadenylate cyclase (DAC) domain of DisA via condensation of two ATP molecules. Witte *et al.* (5) suggested that c-di-AMP is involved in signaling DNA damage considering that the DNA integrity scanning protein DisA scouts the chromosome for DNA double-stranded breaks. Subsequent genomic mining revealed that the DAC domain proteins are widespread in bacteria and archaea, with many of them associated with putative sensor domains (6). The wide occurrence and domain architecture of the DAC domain proteins hinted that c-di-AMP may be another hidden nucleotide messenger mediating various cellular functions and phenotypes.

Currently, there is no report of c-di-AMP degrading or exporting proteins for controlling cellular c-di-AMP level. To identify potential c-di-AMP degrading proteins, we searched bacterial genomes for phosphodiesterase or phosphoesterase proteins that co-occur with the DAC domain-containing proteins (6). We found that a group of multidomain proteins seems to co-occur with a subset of the DAC domain proteins, which include the homologs of YojJ and YbbP from *B. subtilis*. This group of proteins (COG3387), as represented by the *B. subtilis* protein YybT, contains two N-terminal transmembrane helices, a region that shares minimum sequence homology with some Per-Arnt-Sim (PAS) domains, a highly modified GGDEF domain, and a DHH/DHHA1 domain (see Fig. 1). We were particularly attracted to this family of proteins because the DHH domain proteins are known as phosphatases or phosphoesterases for hydrolyzing a wide variety of substrates ranging from inorganic pyrophosphate to single-stranded (ss) DNA (7). The DHH family proteins, which were named after the conserved Asp-His-His motif in the active site, are divided into two subfamilies based on the C-terminal subdomain. Subfamily 1 contains a DHHA1 domain at the C terminus, as represented by the single-stranded DNA exonuclease RecJ and 3′-phosphoadenosine 5′-phosphate (pAp) phosphatase (2, 8). Subfamily 2 contains a DHHA2 domain at the C terminus, as exemplified by the type II inorganic pyrophosphatase (9), yeast cytosol exopolyphosphatase (10), and the eukaryotic c(A/G)MPase protein Prune (11). The C terminus of YybT belongs to the DHHA1 subfamily because it contains the conserved GGGH motif and shares moderate sequence homology with the C termini of RecJ and pAp phosphatase (7). YybT homologs can be identified in pathogens such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Clostridium difficile*. Interestingly, the DHH/DHHA1 domain of the YybT family protein (GdpP)

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S8.

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[2] The abbreviations used are: c-di-GMP, cyclic di-GMP; c-di-AMP, cyclic di-AMP; DAC, diadenylate cyclase; DGC, diguanylate cyclase; pApA, 5′-phosphoadenylyl-(3′→5′)-adenosine; pGpG, 5′-phosphoguanylyl-(3′→5′)-guanosine; PAS, Per-Arnt-Sim domain; ppGpp, tetraphosphate guanosine; ss, single-stranded; pAp, 3′-phosphoadenosine 5′-phosphate; HPLC, high pressure liquid chromatography; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; CHES, 2-(cyclohexylamino)ethanesulfonic acid; AMPPNP, adenosine 5′-(β,γ-iminotriphosphate).

in *S. aureus* has been speculated to be a c-di-GMP hydrolyzing phosphodiesterase domain (12).

In this report, we present results from the study of the cytoplasmic portion of YybT from *B. subtilis*. We demonstrate that the DHH/DHHA1 domain exhibited *in vitro* phosphodiesterase activity toward the cyclic dinucleotides c-di-AMP and c-di-GMP among potential physiological substrates. We present biochemical and bioinformatic evidence to argue that the physiological substrate could be c-di-AMP. We demonstrate that the stringent alarmone ppGpp is a strong competitive inhibitor of the DHH/DHHA1 domain. In addition, we show that the GGDEF domain of YybT possesses unprecedented ATPase activity rather than diguanylate cyclase (DGC) activity. The biological function of YybT and the evolutionary relationships of the DHH/DHHA1 and GGDEF domains are discussed in light of the enzymatic activities and the effect of Δ*yybT* mutation on DNA damage and acid resistance.

EXPERIMENTAL PROCEDURES

Chemicals were purchased from Sigma with the exception of sodium tripolyphosphate (Alfa Aesar), ssRNA and DNA (Eurogentec), c-di-AMP (BioLog), ppGpp (TriLink), and c-di-GMP, which is synthesized using the thermophilic DGC enzyme in the lab (13). The proteins were cloned, expressed, and purified following standard producer as described in the supplemental material.

Genomic DNA Isolation—B. subtilis strain 168 was grown in LB medium. The cells from 5 ml of overnight culture were pelleted and resuspended in Tris-EDTA buffer. The suspension was incubated for 1 h with 6% volume of 10% SDS and 0.6% volume of 20 mg/ml proteinase K at 37 °C. After incubation, an equal volume of phenol/chloroform was added to the mixture. The DNA phenol mixture was spanned at 14,000 rpm for 2 min. The upper aqueous phase was mixed with an equal volume of phenol/chloroform and centrifuged again. A ⅒ volume of sodium acetate (3 M, pH 5.2) was then added to the upper aqueous phase, followed by 0.6 ml of isopropanol to precipitate the genomic DNA. DNA was pelleted by centrifugation and washed by using 70% ethanol. Washed DNA was redissolved in TE buffer for PCR cloning of the YybT gene.

Protein Cloning, Expression, and Purification—The gene (*yybT*) that encodes the full-length YybT was first amplified by PCR from the genomic DNA using the Expand high fidelity kit (Roche Applied Science). Subsequently, the DNA fragments that encode the cytoplasmic portion of YybT ($YybT_{84-659}$), the GGDEF-DHH/DHHA1 di-domain ($YybT_{150-659}$), and the PAS-GGDEF di-domain ($YybT_{84-303}$) were cloned into the expression vector pET-28(a+) (Novagen) between the NdeI and XhoI restriction sites. The plasmids harboring the DNA constructs and the His_6 tag encoding sequence were transformed into *Escherichia coli* strain BL21(DE3).

For protein expression, 1 liter of bacterial culture (LB medium) was grown up to OD = 0.8 before inducing with 0.8 mM isopropyl β-D-thiogalactopyranoside. The culture was kept shaking at 16 °C for ~12 h before it was pelleted by centrifugation. The cells were lysed in 20 ml of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5% glycerol, 0.1% β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride). After the centrifugation at 25,000 rpm for 30 min, the supernatant was filtered and then incubated with 2 ml of nickel-nitrilotriacetic acid resin (Qiagen) for 1 h at 4 °C. The resin was washed with 50 ml of W1 buffer (lysis buffer with 20 mM imidazole) and 20 ml of W2 buffer (lysis buffer with 50 mM imidazole). The proteins were eluted using a step gradient method with elution buffers containing 50 mM Tris, pH 8.0, 150 mM NaCl, 5% glycerol, and 200, 300, or 500 mM imidazole. After the analysis by SDS-PAGE, fractions with purity higher than 95% were pooled together. Size exclusion chromatograph was carried out at 4 °C using the AKTA fast protein liquid chromatography system equipped with a Superdex 200 HR 16/60 column (Amersham Biosciences). The buffer used for gel filtration was comprised of 50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 5% glycerol. The proteins were stored in a −80 °C freezer after flash freezing, and concentration measurement was by the Bradford assay method.

All of the protein mutants were generated using a site-directed mutagenesis II kit (Stratagene) according to the manufacturer's instruction manual. The mutations were verified using a BigDye Terminator v3.1 cycle sequencing kit on ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The mutant proteins were expressed, purified, and stored under the same conditions as wild type.

Bioinformatics and Structure Modeling—Sequence and genomics analysis was performed using STRING and ClustalW (14, 15). In total, 184 YybT homologs (defined as proteins that share the same domain architectures) are found in bacterial genomes. The hidden Markov models (HMM) logo was generated from the alignment file. The structural model of the DHH_{YybT} was built using Swiss-Model sever with the structure of a DHH family protein (Protein Data Bank entry 3DMA) as template (16).

Substrate Screening by Enzymatic Activity Assays—p-Nitrophenol-related substrates *p*NPP, bis-*p*-nitrophenol phosphate, thymidine monophosphate *p*-nitrophenol ester, and phosphenolpyruvate were assayed by monitoring the formation of *p*-nitrophenol at 410 nm using a UV-visible spectrophotometer. A control with the protein storage buffer but not the enzyme solution was used for base-line correction. The enzymatic activity assays with nucleotides and oligonucleotides were carried out by using a HPLC system (Agilent LC1200) with a RP C-18 column as previously described for c-di-GMP activity assay (28, 29). The nucleotides and oligonucleotides tested are listed in supplemental Table S1. The activity toward RNA and ssDNA was monitored by detecting the possible products 1-mer and 2-mer nucleotide along with standards. For inorganic pyrophosphate and polyphosphate, the assay was conducted using the EnzCheck phosphate assay kit (Invitrogen) by measuring the release of P_i in solution. The assay conditions for substrate screening were: 100 mM Tris, pH 8.3, 20 mM KCl, 0.5 mM $MnCl_2$, 1–2 μM enzyme, and 100 μM substrates (10 μM for 24-mer RNA and T7 promoter ssDNA and 1 mM for pyrophosphate and tripolyphosphate). The reactions were incubated for up to 2 h for detecting enzymatic activity.

Metal and pH Dependence—The assay conditions used for metal screening were: 100 mM Tris, pH 8.3, 20 mM KCl, 10 mM [$Metal^{2+}$], 1 μM enzyme, 20 μM c-di-AMP. For pH screening, assay buffer contains 100 mM bis-Tris (for pH 6.7–7.3), Tris (for

pH 7.3–8.8), or CHES (pH 8.8–10) with 0.5 mM $MnCl_2$ and 20 mM KCl.

Kinetic Measurement of the DHH Domain Activity—The measurement of steady-state kinetic parameters were carried out by monitoring the formation of 5′-pApA/5′-pGpG (for DHH/DHHA1 domain) using HPLC. The assay buffer conditions are the same as described above. Initial velocity at a certain substrate concentration was obtained from a series of reactions that were stopped at various time points within linear range. Initial velocity was measured at seven or eight substrate concentrations. The kinetic parameters k_{cat} and K_m were obtained by fitting the initial velocity at various substrate concentrations to a Michaels-Menten equation using the software Prism (GraphPad). For ppGpp inhibition assay, the initial velocity for c-di-AMP hydrolysis (at 20 μM) was first measured in the presence of 0–2 mM ppGpp under the same reaction conditions used for steady-state kinetic measurement. The IC_{50} was obtained by fitting the data to the dose-response model using the software Prism (GraphPad). The steady-state kinetics in the presence of two ppGpp concentrations was measured with K_I obtained from data fitting.

Kinetic Measurement of the ATPase Activity—The ATPase activity of the GGDEF domain was assayed by two methods. The hydrolysis of ATP was monitored by measuring the release of P_i in solution using the EnzCheck phosphate assay kit (Invitrogen) and a UV-visible spectrophotometer or directly monitoring ADP formation using HPLC with a solvent system described by Ryjenkov *et al.* (17). The measurement of steady-state kinetic parameters was carried out by monitoring the formation ADP using HPLC. All of the kinetic measurements were carried out at 23 °C with the assay conditions: 50 mM Tris, pH 7.5, 1 mM $MgCl_2$, 0.05–8 mM ATP, 2.9 μM enzyme. Competitive inhibition was carried out in the presence of 250 μM AMPPNP. The dissociation constants (K_D) were calculated using the equation: $K_D = [\text{Inhibitor}]/(K_m(I)/K_m - 1)$.

Acid Stress Resistance—The procedure was adopted from a previous report (18). The wild type and Δ*yybT* mutant *B. subtilis* cells (obtained from Prof. K. Kobayashi, Nara, Japan) grown from an overnight culture at pH 7.0 were inoculated into LB medium (pH 4.0, 3.5, or 3.0) at final $A_{600\ nm} = 0.1$. To determine viability by colony formation, the cells were plated on LB agar plate (pH 7.0) with proper dilution both prior to (T0) and after acid challenge for 2 h (T2). Relative survival was calculated as the ratio of colony-forming units/ml at time T2 to that at T0.

Sporulation of Wild Type and ΔyybT Mutant in the Presence of Nalidixic Acid—The spore formation efficiency of the wild type *B. subtilis* strain 168 and YybT mutant strain (Δ*yybT*) were examined by using an established procedure (4). ΔYybT was obtained from a complete mutant library of *B. subtilis* 168 with an insertion of pMUTIN3MCS after the codon for amino acid 88 (19). Growth and sporulation were carried out with shaking at 32 °C in casein hydrolysate medium and sporulation medium, respectively (4, 37). Sporulation was induced by transferring the early log-phase cells from the casein hydrolysate medium to the sporulation medium with the DNA double break inducing nalidixic acid (sigma) at the indicated concentration. After 22 h, the cells were heated at 80 °C for 10 min for spore selection and then plated with proper dilutions on LB agar plates (number of colonies < 500/plate). The whole experiment was repeated three times to ensure reproducibility. The spore formation efficiency was estimated by calculating the percentage of spores, which equals (number of colonies with heating)/(number of colonies without heating) × 100.

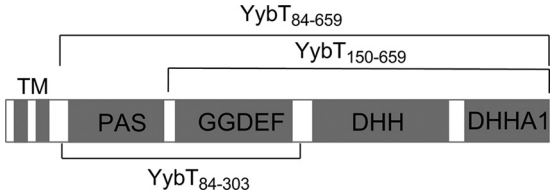

FIGURE 1. **Domain architecture of YybT family proteins (COG3387) with the three protein constructs studied in this work indicated.**

Measurement of the Cellular c-di-AMP Concentration—The procedure for measuring the cellular concentration of c-di-AMP in *B. subtilis* was essentially the same as the one reported by Simm *et al.* (20). Briefly, the vegetative and sporulating cells harvested from the casein hydrolysate and sporulation medium were pelleted and resuspended in double distilled H_2O (100 mg of cells/300 μl of water). The suspension was heated at 95 °C for 15 min, followed by sonication for 5 min. 99% ethanol was then added to the sample to a final concentration of 70%. After centrifugation, the supernatant was pooled, frozen, and subsequently lyophilized overnight. The precipitate was dissolved in 500 μl of double distilled H_2O, filtered, and loaded to the HPLC system. Because no clear c-di-AMP peak was observed on HPLC trace, a 4-min fraction corresponding to the retention time of c-di-AMP (±2 min) was collected from HPLC, lyophilized, and redissolved into 5–10 μl of double distilled H_2O. For mass spectrometry, the sample was mixed with an equal volume of matrix (α-cyano-4-hydroxycinnamic acid, 20 mg/ml) before being spotted onto 384-well plate to crystallize. Mass spectra were recorded with a 4800 matrix-assisted laser desorption ionization time-of-flight analyzer (Applied Biosystems) in both positive and negative modes.

RESULTS

Three protein constructs with different domain combination were cloned, expressed, and purified as His_6-tagged proteins (Fig. 1). $YybT_{84-659}$ contains the whole cytoplasmic portion, whereas $YybT_{150-659}$ and $YybT_{84-303}$ contain the GGDEF-DHH/DHHA1 and PAS-GGDEF segments, respectively. The recombinant proteins were purified by nickel-nitrilotriacetic acid affinity and size exclusion chromatography to homogeneity (supplemental Fig. S1). The purified proteins were not bound by RNA, DNA, or small nucleotide ligands as detected by HPLC and spectroscopic analysis of the denatured protein solution.

The DHH/DHHA1 Domain of YybT Exhibits Specific Phosphodiesterase Activity—We first tested the activity of YybT toward four *p*-nitrophenol substrates that are known to be easy substrates for phosphodiesterases and phosphatases. In the presence of Mg^{2+} or Mn^{2+}, $YybT_{84-659}$ hydrolyzed bis-*p*-nitrophenol phosphate and thymidine monophosphate *p*-nitrophenol ester but not *p*-nitrophenol phosphate and phosphenolpyruvate (Fig. 2*B*), indicating that the enzyme is a phosphodiesterase rather than a phosphatase. This conclusion

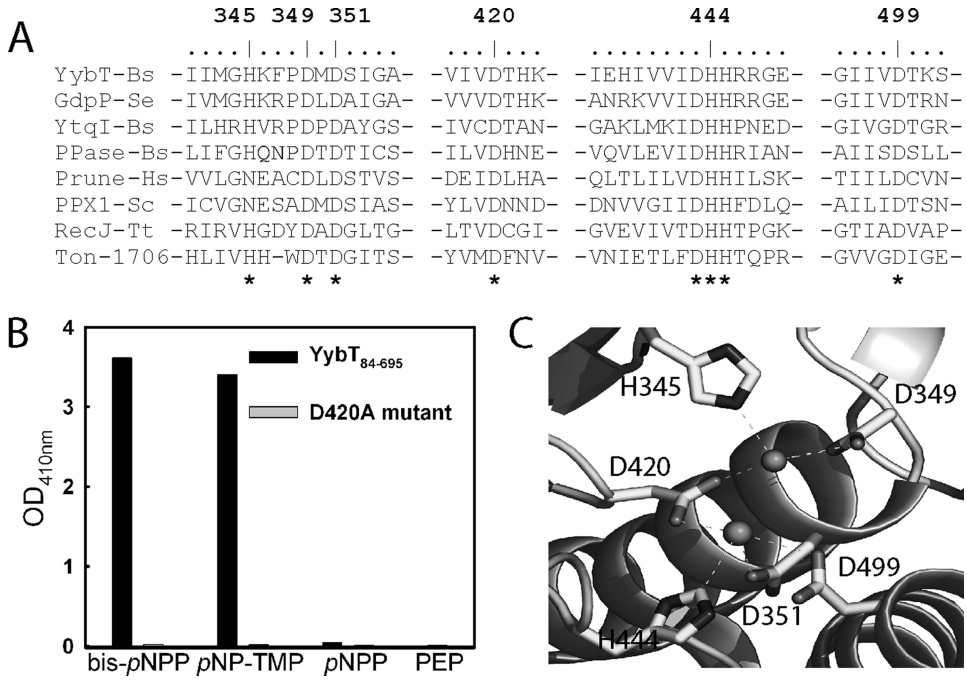

FIGURE 2. **Specific phosphodiesterase activity of YybT$_{84-659}$.** *A*, partial alignment of DHH-DHHA1 domain sequences with the conserved residues for metal ion coordination highlighted. *B*, enzymatic activity of YybT against nonphysiological *p*-nitrophenol substrates. *C*, structural model of DHH$_{YybT}$ active site with the metal ions shown as *balls* and the coordinating residues as *sticks*.

is further supported by the observation that YybT did not hydrolyze monophosphate ribonucleotides and deoxyribonucleotides (supplemental Table S1). Moreover, YybT did not act on 3′-phosphoadenosine 5′-phosphate (PAP), suggesting that YybT differs from the DHH family phosphatase YtqI (21). YybT also did not exhibit activity toward sodium tripolyphosphate and pyrophosphate, excluding it as a pyro- or polyphosphatase (10, 22, 23).

After ruling out YybT as a phosphatase, we examined the activity of YybT toward a wide range of possible physiological substrates for phosphohydrolyase or phosphodiesterase as listed in supplemental Table S1. Di- and tri-phosphate nucleotides, deoxyribonucleotides, and NADH, NADPH, NAD^+, $NADP^+$, and FAD were first tested. No phosphohydrolyase activity was observed for all of the compounds with the exception of ATP. We found that ATP was slowly converted to ADP. However, this activity was attributed to the GGDEF domain rather than the DHH domain as we will discuss later. We next tested whether YybT functions as an oligoribonuclease or ribonuclease for degrading RNA or ssDNA (21, 24). Neither the ssDNA and RNA substrate nor the 16 oligoribonucleotides and oligodeoxyribonucleotides of various lengths could be degraded by YybT in the presence of Mn^{2+} or Mg^{2+}. Moreover, Yybt did not degrade diadenosine tetraphosphate (AppppA), triphosphate (ApppA), or pentaphosphate (AppppppA), suggesting that it is not a bis(5′-adenosyl) tri-, tetra-, or pentaphosphatase (25, 26). Lastly, the small nucleotide (p)ppGpp is known as a bacterial and plant messenger for signaling starvation and other stresses (27). The enzymatic assay showed that YybT did not degrade ppGpp and thus is not a ppGpp-specific phosphohydrolyase or phosphatase.

Several naturally occurring cyclic nucleotide substrates were tested for phosphodiesterase activity. No activity was detected with 3′,5′-cAMP and cGMP even after prolonged incubation with Mg^{2+} or Mn^{2+}. In contrast, the incubation of YybT with c-di-AMP and c-di-GMP resulted in the disappearance of the cyclic dinucleotides and the formation of new products. Based on HPLC analysis, YybT hydrolyzes c-di-AMP and c-di-GMP to produce exclusively the linear dinucleotides 5′-pApA and 5′-pGpG, without generating the 3′-dinucleotide (3′-pApA or 3′-pGpG) or monophosphate (AMP or GMP) (Fig. 3*A* and supplemental Figs. S2 and S4). Steady-state kinetic measurement yielded a turnover number (k_{cat}) of $0.55 \pm 0.02\ s^{-1}$ and Michaelis-Menten constant (K_m) of $1.3 \pm 0.3\ \mu M$ for c-di-AMP (Fig. 3*B*). In comparison, YybT exhibited a comparable k_{cat} ($0.23 \pm 0.02\ s^{-1}$) but a much greater K_m ($349 \pm 55\ \mu M$) for c-di-GMP. The difference in K_m indicates that the substrate-binding pocket prefers c-di-AMP over c-di-GMP. The cyclic dinucleotide specific activity of YybT could also be detected with the construct (YybT$_{150-659}$) that contains the GGDEF-DHH/DHHA1 segment, although YybT$_{150-659}$ exhibited a ~10-fold decrease in k_{cat} (supplemental Table S2). According to sequence comparison and the structural model of the DHH$_{YybT}$ subdomain, the residues Asp^{420} and Asp^{499} are predicted to be the metal ion-coordinating residues (Fig. 2, *A* and *C*). The D420A mutant only exhibited residual activity against c-di-AMP, c-di-GMP, bis-*p*-nitrophenol phosphate, and thymidine monophosphate *p*-nitrophenol ester (Fig. 2*B*), whereas the double mutation D420A/D499A totally abolished the catalytic activity. These observations confirm that the DHH domain is responsible for the specific phosphodiesterase activity.

Metal and pH Dependence of the Phosphodiesterase Activity—The phosphodiesterase activity of YybT is strictly dependent on divalent metal ions such as Mn^{2+}, Mg^{2+}, Ni^{2+}, and Co^{2+} ions (Fig. 4*A*). Meanwhile, Zn^{2+} ion inhibits catalytic activity in the presence of Mn^{2+} or Mg^{2+}, whereas Ca^{2+} only inhibits catalysis in the presence of Mg^{2+} and not Mn^{2+} (Fig. 4*B*). Mn^{2+} and Mg^{2+} are most likely the physiological metal ion for YybT, considering that DHH family proteins tend to use Mn^{2+} or Mg^{2+} for catalysis (24). We examined the Mg^{2+} and Mn^{2+} ion dependence of the catalytic activity for identifying the physiological metal ion for YybT (Fig. 4*C*). Mn^{2+} ion exhibits a bell-shaped profile with the optimal contraction in the range of 10 μM to 10 mM, with the activity strongly inhibited at high [Mn^{2+}] concentration (≥10 mM). By contrast, the catalytic activity remained low for Mg^{2+} at 1 mM [Mg^{2+}] and did not increase significantly until [Mg^{2+}] reached 10 mM. Because the cellular

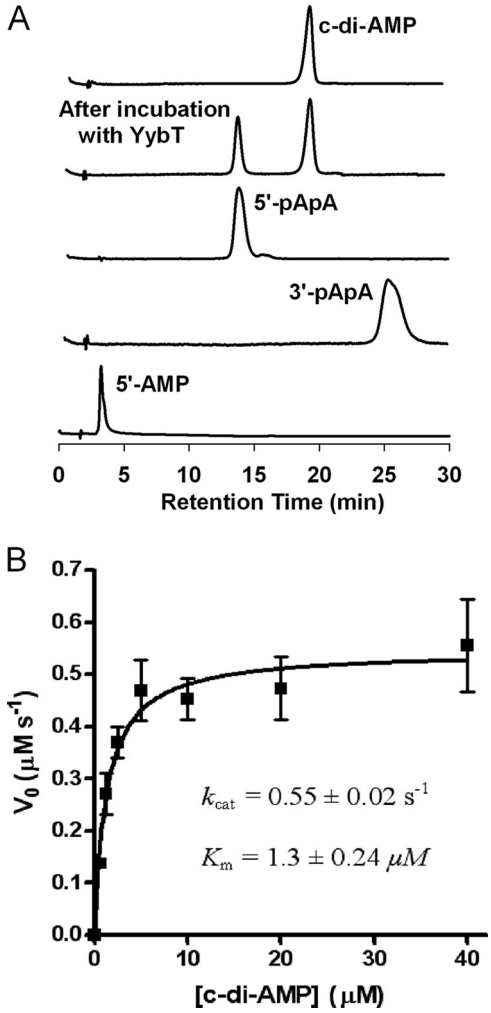

FIGURE 3. **Degradation of c-di-AMP by YybT.** *A*, HPLC analysis of the hydrolysis of c-di-AMP by $YybT_{84-659}$. The assay conditions are described under "Experimental Procedures". *B*, steady-state kinetic analysis of the hydrolysis of c-di-AMP catalyzed by $YybT_{84-659}$.

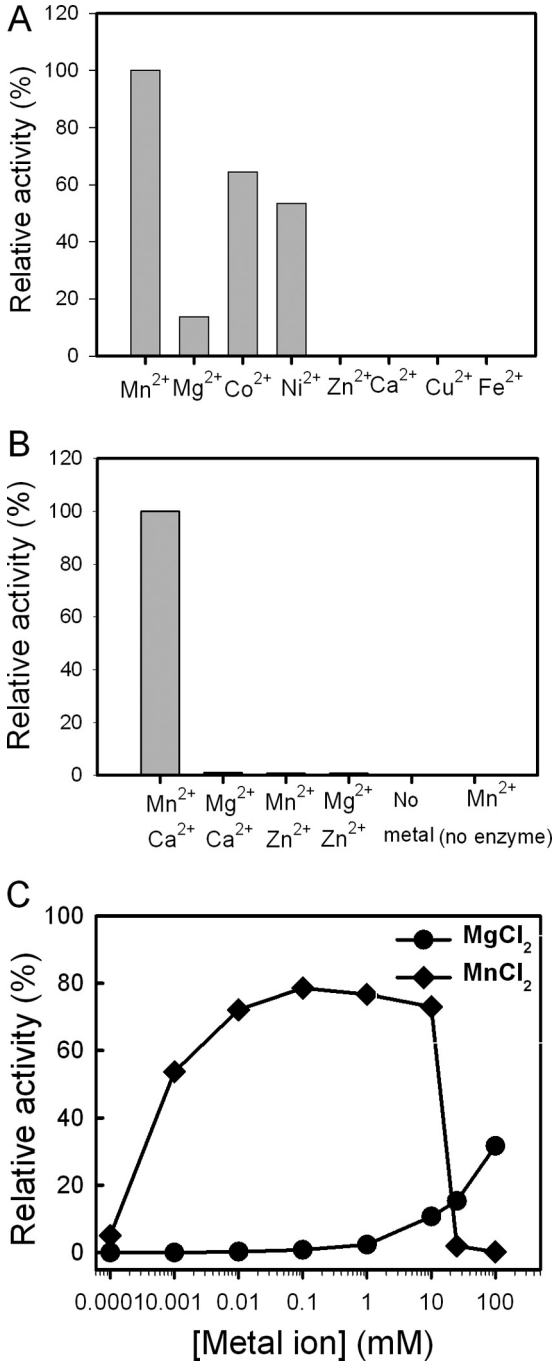

FIGURE 4. **Metal and pH dependence of c-di-AMP degradation by $YybT_{84-659}$.** *A*, metal dependence of YybT-catalyzed c-di-AMP hydrolysis. The enzymatic assay conditions are described under "Experimental Procedures." *B*, Mn^{2+} and Mg^{2+} concentration dependence of c-di-AMP hydrolysis. *C*, pH dependence of DHH_{YybT} domain activity.

Mn^{2+} and Mg^{2+} concentrations are approximately 10 μM and 1 mM, respectively, in *B. subtilis* (28), the results support Mn^{2+} ion as the physiological metal ion for the DHH/DHHA1 domain of YybT. In addition, the pH dependence study showed a strong preference for alkaline conditions with the optimal pH between 8.5 and 9.0 (supplemental Fig. S3), which is in line with a two-metal ion-assisted catalytic mechanism (29).

The GGDEF Domain of YybT Binds and Hydrolyzes ATP—Initial enzymatic assays suggested that the GGDEF domain of YybT is neither a functional DGC domain for converting GTP into c-di-GMP nor a GTPase for hydrolyzing GTP. During substrate screening for the DHH/DHHA1 domain of YybT, we noticed that the concentration of ATP decreases with time in the presence of YybT and Mg^{2+} (or Mn^{2+}). HPLC analysis revealed that the depletion of ATP was accompanied by the formation of ADP, suggesting that YybT catalyzes the hydrolysis of ATP. Surprisingly, the D420A/D499A double mutation in the DHH domain did not affect ATP hydrolysis, hinting that DHH/DHHA domain is not responsible for the ATPase activity. Meanwhile, sequence alignment showed that YybT contains a highly modified GGDEF domain, which lacks the signature GGDEF motif and the guanosine-binding residues critical for the DGC activity (30). To test whether the degenerate GGDEF domain is responsible for the ATPase activity, we cloned and expressed another protein construct ($YybT_{84-303}$) that only contains the PAS-GGDEF didomain. Although the purified $YybT_{84-303}$ protein did not show any activity against GTP, it could still hydrolyze ATP to yield ADP exclusively (Fig. 5*A*). An enzymatic assay with the EnzCheck phosphate assay kit confirmed that the inorganic phosphate was released into the

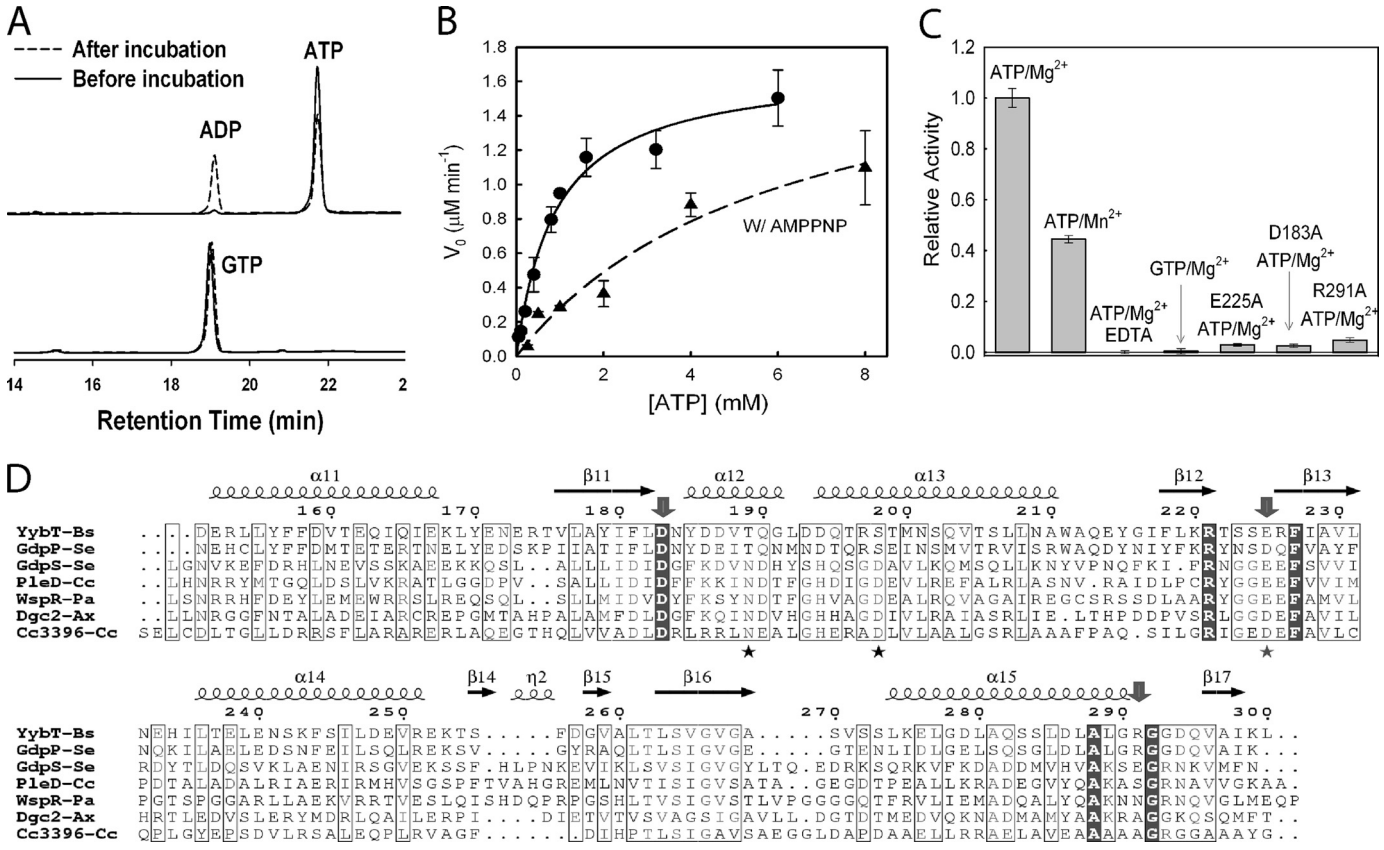

FIGURE 5. **ATPase activity of the GGDEF$_{YybT}$ domain.** *A*, HPLC analysis of ATP hydrolysis catalyzed by YybT$_{84-303}$. *B*, steady-state kinetic measurement of ATPase domain activity in the presence and absence of nonhydrolyzable ATP analog. *C*, relative ATPase activity of YybT$_{84-303}$ and its mutants in the presence of Mg^{2+} and Mn^{2+}. *D*, sequence comparison of the GGDEF$_{YybT}$ domain with some characterized GGDEF domains. The three residues mutated in this study are indicated by the *vertical arrows*, whereas the residues for guanosine binding in orthodox GGDEF domains are indicated by *asterisks*. GdpP (*S. aureus*) is a homolog of YybT. The GGDEF domain of GdpS (*S. aureus*) exhibits residual GTPase activity. The GGDEF domains of PleD (*C. crescentus*) (30), WspR (*P. aeruginosa*) (54), and *Ax*DGC2 (*Acetobacter xylinum*) function as diguanylate cycles (55). The GGDEF domain of Cc3396 binds GTP as a regulatory domain.

solution, suggesting that the GGDEF is not a kinase that transfers the phosphate onto a substrate (supplemental Fig. S7). Steady-state kinetic measurement yielded a k_{cat} of 0.59 ± 0.03 min^{-1} and K_m of 0.90 ± 0.12 mM for the ATPase in the presence of Mg^{2+} ion (Fig. 5*B*). The metal ion Mg^{2+} or Mn^{2+} is required for the ATPase activity with Mg^{2+} as the preferred metal ion, in contrast to the metal preference for the DHH/DHHA1 domain (Fig. 5*C*). To further provide evidence for the preferred binding of ATP by the divergent GGDEF domain, we measured the dissociation constant (K_D) for the nonhydrolyzing analog of ATP (adenylyl imidodiphosphate AMPPNP) by competitive inhibition experiments (Fig. 5*B*). The measurement revealed a K_D of 44.0 ± 14.6 μM for AMPPNP. The presence of the AMPPNP did not exert any significant effect on the DHH/DHHA1 domain activity, suggesting that the GGDEF domain is not an ATP-binding regulatory domain. Finally, although the signature GGDEF motif and the residues for guanosine group binding are absent, the residues that coordinate the Mg^{2+} ions in orthodox GGDEF domains, with one in the signature GG(D/E)EF motif and another at the end of β-strand 1, seem to be conserved in GGDEF$_{YybT}$ (Glu^{225} and Asp^{183}) (31, 32) (Fig. 5*D* and supplemental Fig. S6, *A* and *B*). We found that the mutation of the conserved residues Asp^{183}, Glu^{225}, and the conserved γ-phosphate binding Arg^{291} reduced the catalytic activity to below 5% (Fig. 5*C*). Although the structural motifs for the binding of the adenosine group remain to be defined, the mutagenesis results suggested that the metal ion and phosphate-binding sites in the GGDEF domain may be preserved despite the divergence of function.

Inhibition of the Phosphodiesterase Activity by ppGpp—Screening of *Lactococcus lactis* mutants revealed that the YybT family protein llmg1816 is involved in acid and starvation stress resistance, with the Δ*llmg1816* mutant strain exhibiting a higher survival rate under low pH and starvation conditions (18). It was postulated that the deletion of llmg1816 may cause change in the (p)ppGpp level (18). (p)ppGpp is a global messenger that plays a major role coordinating cellular responses under starvation stresses. The level of (p)ppGpp is significantly elevated during stringent responses, resulting in the binding of (p)ppGpp to its protein targets such as RNA polymerase and inosine monophosphate dehydrogenase (18, 33, 34).

To test whether the YybT protein is a previously unknown target for (p)ppGpp, the phosphodiesterase and ATPase activities were examined in the presence of ppGpp. Remarkably, although ppGpp did not inhibit or stimulate the ATPase activity, it significantly suppressed the c-di-AMP hydrolyzing activity of the DHH/DHHA1 domain. An apparent IC_{50} value of 234 μM was derived from the activity *versus* [ppGpp] plot (Fig. 6*A*). Initial velocity measurement at various ppGpp concentrations indicated that the inhibition is competitive in nature, with an

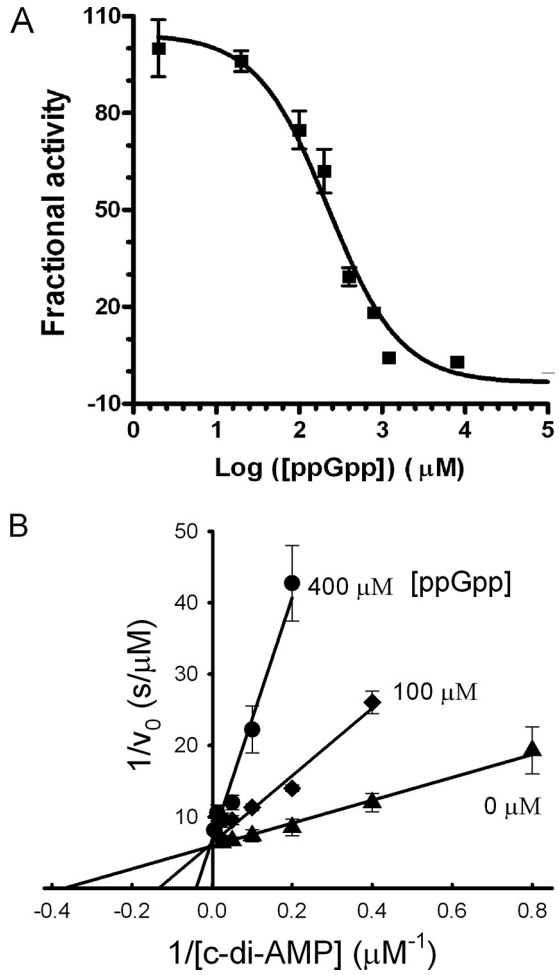

FIGURE 6. **Inhibition of the phosphodiesterase activity by ppGpp.** *A*, ppGpp concentration dependence of the c-di-AMP hydrolyzing activity. The IC_{50} value is determined from the midpoint of the plot. *B*, double-reciprocal plots of c-di-AMP hydrolyzing activity at various ppGpp concentrations. The experimental conditions are described under "Experimental Procedures."

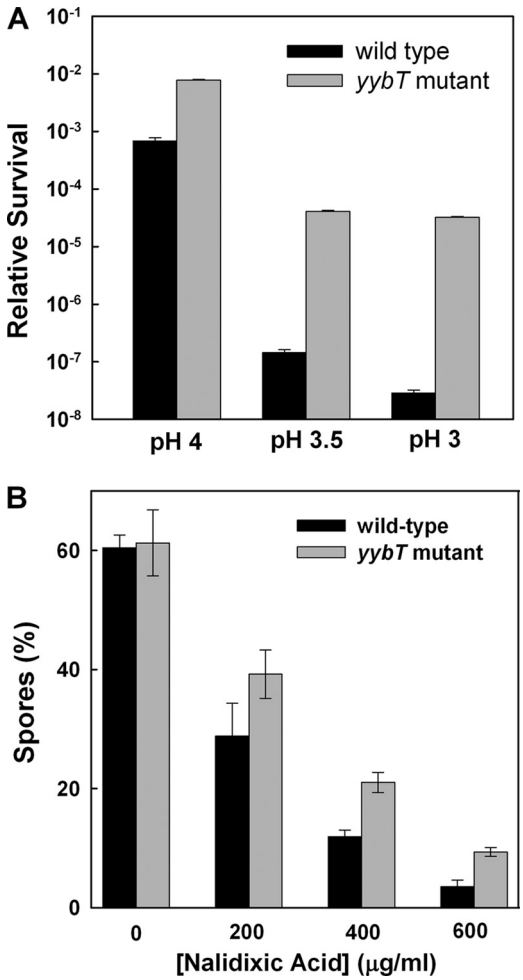

FIGURE 7. **Acid and DNA damage resistance.** *A*, relative survival of wild type and Δ*yybT* strain under acid stress conditions. *B*, spore formation efficiency for the wild type *B. subtilis* and Δ*yybT* mutant in the presence of DNA damage-inducing agent nalidixic acid. The spores (%) were obtained as described under "Experimental Procedures."

inhibition constant (K_I) of 35.9 ± 7.2 μM (Fig. 6*B*). Considering that bacterial cells can rapidly accumulate ppGpp up to a millimolar level under starvation conditions (27, 35), the inhibition of the phosphodiesterase activity by ppGpp is likely to be physiologically relevant. No such inhibition effect was observed with other cyclic or linear nucleotides or on the c-di-GMP hydrolyzing activity of the EAL domain proteins such as RocR (36, 37), indicating that the inhibition of the DHH/DHHA1 domain by ppGpp is rather specific.

Effect of ΔyybT Mutation on Acid and DNA Damage Resistance—L. lactis contains an *yybT*-like gene (*llmg1816*). The Δ*llmg1816* mutant was found to be more resistant under acid stress and also to exhibit enhanced long term (2-day) survival (18). We found that the Δ*yybT* mutant strain of *B. subtilis* is also more resistant than the wild type against acid stress (Fig. 7*A*). Because the c-di-AMP synthesizing DisA has been proposed to signal DNA damage (5), we further tested whether YybT plays a role in mediating DNA damage resistance. No significant survival advantage or disadvantage was observed when the wild type *B. subtilis* and Δ*yybT* mutant were treated with the topoisomerase inhibitor nalidixic acid (DNA gyrase inhibitor) or γ-ionizing irradiation (supplemental Fig. S8), suggesting that YybT does not play a significant role in DNA repair during vegetative growth. However, when we compared the sporulating cells, a significant difference was observed between the wild type and mutant strains. As shown in Fig. 7*B*, when both the wild type and *yybT* mutant cells were induced to enter sporulation without exposure to nalidixic acid, the spore formation efficiency was comparable, suggesting that the gene knock-out does not significantly affect sporulation. However, in the presence of nalidixic acid, the mutant strain consistently exhibited more colonies, which were derived from the spores that survive the heat treatment. The discrepancy in spore formation efficiency widens between the wild type and the *yybT* mutant with increasing nalidixic acid concentration. This is in sharp contrast to the observation for the Δ*disA* mutant, which formed less spores than the wild type strain (4). These observations indicate that YybT plays a role in DNA damage signaling and repair during sporulation.

Measurement of the Cellular c-di-AMP Concentration—To test whether YybT affects global c-di-AMP level, we set out to measure the cellular concentration of c-di-AMP for the wild type and Δ*yybT* strains during vegetative growth and sporulation. Intriguingly, although we could readily detect the c-di-

AMP standard in the spiked sample as well as c-di-GMP in *E. coli* and *Pseudomonas aeruginosa* by using the mass spectrometry method described by Simm *et al.* (20), we could not observe any c-di-AMP peak above the background in both the wild type and the mutant strains after repeated attempts. The results led us to suspect that the steady-state concentration of c-di-AMP in *B. subtilis* is rather low in bacterial cells.

DISCUSSION

DHH family proteins function as phosphatase or phosphodiesterases for hydrolyzing a wide variety of substrates that range from pyrophosphate to ssDNA. The enzymatic assay results suggested that YybT possesses different substrate specificity from other known DHH family proteins. The biochemical assay revealed that YybT is a novel phosphodiesterase that hydrolyzes the cyclic dinucleotides c-di-GMP and c-di-AMP. The substrate specificity is consistent with the low sequence homology shared by the substrate-binding DHHA1 subdomains between YybT and other DHH family proteins. c-di-GMP is a ubiquitous second messenger in bacteria and is known to be degraded by the EAL domain and HD-GYP domain phosphodiesterases (38, 39). Although c-di-GMP could be degraded by YybT in our assay, it is unlikely to be the physiological substrates for YybT for two major reasons. First, the cellular c-di-GMP concentration is in the low or submicromolar range according to the estimated cellular c-di-GMP concentration (40–42). In addition, the K_m values for the c-di-GMP degrading EAL domain proteins are also known to be in the same range (36, 37, 43). Hence, the elevated K_m (349 ± 54.6 μM) for c-di-GMP implies that the DHH/DHHA1 domain is incapable of hydrolyzing c-di-GMP efficiently under physiological conditions. Second, considering that the YybT family proteins (COG3387) can be found in the bacteria (*e.g. Streptococcus* and *Staphylococcus*) that do not seem to harbor c-di-GMP signaling network (as judged by the absence of catalytically active EAL/HD-GYP and GGDEF domain proteins (12)), the YybT family proteins are unlikely to be involved in c-di-GMP signaling. Hence, the physiologically irrelevant K_m and the presence of YybT in bacteria that lack the c-di-GMP network strongly disfavor c-di-GMP as the cellular substrate for YybT.

On the other hand, the biochemical and genetic data seem to support c-di-AMP as the cellular substrate for YybT. First, the small K_m of 1.3 ± 0.3 μM toward c-di-AMP indicates that YybT can degrade c-di-AMP efficiently even if each *B. subtilis* cell only contains 200–600 molecules of c-di-AMP, which corresponds to a cellular concentration in the low or submicromolar range. Second, the fact that YybT hydrolyzes c-di-AMP to generate exclusively 5′-pApA (not 3′-pApA or AMP) suggests that the substrate-binding pocket can differentiate the cyclic dinucleotide and the linear dinucleotide (*i.e.* the product pApA). The crystal structures of several DHH domain proteins revealed that the C-terminal subdomain (DHHA1 or DHHA2) is largely responsible for the binding of substrate (10, 24, 44). Sequence alignment of YybT and DHH family proteins revealed that although the DHH subdomain is fairly conserved with all the putative metal ion-binding residues (Fig. 2*A*), the DHHA1 subdomain does not share significant sequence homology with any characterized DHH family proteins. Sequence comparison also revealed that the Arg residues critical for the binding of polyphosphate, RNA, or ssDNA in other DHHA1 domains (*e.g.* RecJ and YtqI) are not conserved in YybT. In contrast, only one conserved Arg (Arg^{606}) was found in YybT homologs (supplemental Fig. S5). This Arg is likely to interact with the phosphate group of c-di-AMP according to our structural model. Thus, the uniqueness of the DHHA1 subdomain of YybT seems to be in agreement with its unprecedented substrate specificity. Third, the only putative phenotype mediated by c-di-AMP is the signaling of DNA damage. The mutation of DisA significantly retards sporulation in the presence of a DNA damaging agent. In sharp contrast, the Δ*yybT* mutant exhibited higher spore formation efficiency than the wild type (Fig. 7). Third, DAC domains can be found in 275 bacterial or archaeal species with many of them belonging to the phylums of firmicutes and ϵ-proteobacteria, whereas YybT family proteins can be found in 123 bacterial species that mainly consist of firmicutes (supplemental Table S3). Importantly, for all the 123 bacterial species that contain the YybT family protein, at least one DAC domain protein can be identified. Based on these considerations, we propose that YybT is probably involved in c-di-AMP signaling by hydrolyzing the cyclic dinucleotide, resembling the role of the EAL domain in c-di-GMP signaling. Meanwhile, the absence of YybT protein in some DAC domain protein-containing bacteria seems to hint of the existence of other families of c-di-AMP-specific phosphodiesterases.

It has been noticed that YybT family proteins contain a highly modified GGDEF domain with unknown function (12, 45). GGDEF domains are homologs of adenylate cyclase domains and are mainly known as DGC domains for synthesizing c-di-GMP from GTP (17, 46). A large number of catalytically inactive GGDEF domains that lack the signature GG(D/E)EF motif have been identified, including two divergent GGDEF domains that can still bind GTP. Jenal and co-workers (43) reported a regulatory GGDEF domain that binds GTP and stimulates the activity of the adjacent EAL domain, whereas O'Gara and co-workers (12) recently reported a GGDEF domain with residual GTPase activity. In contrast to these GGDEF domains, the current study revealed that the GGDEF domain of YybT specifically binds and hydrolyzes ATP but not GTP. Sequence comparison suggested that the metal ion-binding residues in $GGDEF_{YybT}$ are probably conserved, but the residues for the guanosine group binding are absent (Fig. 5*D*). Based on the mutagenesis results, we speculate that ATP is probably bound at approximately the same site as GTP in the typical GGDEF domains, although the structural motifs that confer the binding specificity remain to be identified. Interestingly, phylogenetic analysis revealed that the GGDEF domain of YybT resembles adenylate cyclase domains more than the typical GGDEF domains (supplemental Fig. S6*C*), providing further support for the notion that GGDEF and adenylate cyclase domains were evolved from the same ancestor (47). Considering that the YybT family proteins are only found in an early branch of the evolutionary tree, the ATPase activity exhibited by the $GGDEF_{YybT}$ domain seems to suggest an early divergence of function for the GGDEF domains during evolution. Lastly, the intrinsic ATPase activity of the GGDEF domain is relatively low, reminiscent of the basal activity of the AAA^+-

ATPases prior to the stimulation by small molecule or protein partners (48). It is intriguing to speculate whether the PAS domain may function as a sensor domain for perceiving and transducing a signal to further stimulate the activity of the GGDEF-based ATPase domain.

The nucleotide ppGpp has been known as the stringent alarmone that regulates RNA polymerase and other protein targets for gene expression and other cellular activities during stringent responses (49). The inhibition of the DHH/DHHA1 domain by ppGpp suggests a direct link between the ppGpp and c-di-AMP signaling networks and implies that the local c-di-AMP level will increase during stringent responses to starvation and other stresses. The measured IC_{50} and K_I values indicate that the hydrolysis of c-di-AMP will be fully suppressed during stringent response with the ppGpp level raised above 1 mM. Interestingly, the interference of the ppGpp and c-di-GMP signaling networks has also been proposed recently (50, 51). The inhibition of c-di-AMP hydrolysis by ppGpp may represent another example of cross-talking between two nucleotide signaling networks.

In addition to the novel enzymatic activities exhibited by YybT, the opposite effects of the Δ*disA* and Δ*yybT* mutation on sporulation in the presence of a DNA-damaging agent point toward contrasting roles played by the proteins in DNA damage resistance. The higher sporulation efficiency for the Δ*yybT* mutant indicates that the mutant strain is more resistant against DNA damage, whereas the acid resistance experiment showed that the Δ*yybT* mutant strain is more tolerant to acid stress, consistent with the observation for the *L. lactis* mutant (25). Because acid stress can also cause chromosomal DNA damage in bacteria (52, 53), the observed acid and DNA damage resistance may be highly related on the molecular level. Together, the acid and DNA damage resistance exhibited by the mutant suggest that YybT functions as a signaling protein for coordinating stress sensing and cellular responses. Finally, considering that the DHH/DHHA1 domain of YybT is an efficient c-di-AMP phosphodiesterase under *in vitro* conditions and that the c-di-AMP synthesizing DisA is implicated in signaling DNA damage (5), it is tempting to conclude that the effect of the Δ*yybT* mutation is exerted through c-di-AMP. However, it remains to be fully established in the future whether c-di-AMP is truly a signaling messenger that regulates the phenotypes associated with stress responses. The failure to detect c-di-AMP in both the vegetative and sporulating *B. subtilis* cells seems to hint that even if the cyclic nucleotide is at play, it may only function at a local level.

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